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Correlation analysis of vascular endothelial growth factor level with clinicopathological features and prognosis in patients with diabetic nephropathy: A biopsy-based study

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ABSTRACT

Globally, Type 2 diabetes mellitus (T2DM) is one of the most prevalent chronic diseases, which poses a great potential threat to the human body. Diabetic nephropathy (DN), a very common complication in T2DM, is also the main trigger for end-stage renal disease. A thorough understanding of the pathogenesis is the key as well as the breakthrough for future diagnosis and treatment of DN. This investigation aims to provide more in-depth and accurate guidance for future follow-up research by analyzing the role of vascular endothelial growth factor (VEGF) in the kidney tissue of DN patients. Seventy-nine patients with suspected DN who underwent renal needle biopsy in our hospital from January 2015 to June 2019 were selected as the research participants. After the biopsy, 36 cases were confirmed as DN, and the other 43 were T2DM with primary glomerulonephritis. Determination of VEGF mRNA and protein expression in renal tissue employed PCR and Western blot, and the connection between VEGF mRNA level and clinical pathology (such as renal function, inflammatory factors and pathological manifestations) was discussed. The disease recurrence in DN patients was recorded through the 3-year prognostic followed up, and the related influencing factors were analyzed. In kidney tissue, VEGF mRNA level and protein expression were notably higher in DN patients than in diabetic patients ($P<0.05$). Pearson correlation coefficient analysis identified that VEGF mRNA and protein had a positive connection with blood urea nitrogen (BUN), serum creatinine (Scr), 24-hour urine total protein (24hUTP) and C-reactive protein (CRP) ($P<0.05$). Among the various clinicopathological features of DN patients, age, BMI, sex, family history, smoking, drinking, exercise habits, clinical presentations and pathological changes had no significant relationship with VEGF level ($P>0.05$), but the course of the disease, fasting blood glucose (FBG), glycosylated hemoglobin (HBALC) and pathological stages of nephropathy had a close connection with VEGF level ($P<0.05$). Prognostic follow-up revealed that VEGF mRNA was noticeably higher in patients with recurrence than in those without ($P<0.05$). When VEGF mRNA >5.20 in kidney tissue, the sensitivity and specificity for predicting the 3-year recurrence were 85.71% and 84.00% respectively ($P<0.05$). Finally, Logistic regression analysis identified the independence of FBG, HBALC and VEGF levels as the influencing factors for the prognostic recurrence of DN ($P<0.05$). VEGF expression in kidney tissue of DN patients is closely linked to renal function and increases as the disease progresses, which is an independent risk factor associated with the prognostic recurrence of DN, with great potential significance for future DN diagnosis and treatment.

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Introduction

Pervasive among the middle-aged and the elderly, Type 2 diabetes mellitus (T2DM) is one of the chronic diseases with the highest incidence all over the world (1). According to statistics, there are more than one billion people suffering from T2DM worldwide, and the incidence rate is as high as 6-10% (2, 3). Instead of a complete cure clinically, lifelong blood sugar control can go a long way in helping diabetic patients keep the disease from getting worse (4). And rather than the disease per se, the great threat T2DM poses is through affecting the life and health of patients by inducing various organ and tissue diseases (5). Among them, diabetic nephropathy (DN) is a very common complication in T2DM, accounting for more than 40% of all diabetic patients (6). It can cause hypertrophy and fibrosis of glomeruli and increased extracellular matrix production in patients, which is also one of the main triggers for end-stage kidney disease at present (7). For DN, due to the metabolic disorder of the internal environment, the treatment difficulty is further enhanced (8). In clinical practice, DN patients are usually treated with renal dialysis, but the effect is not ideal, and the 5-year survival rate is only 40%-50% (9, 10).

Clinically, it is pointed out that a thorough understanding of the pathogenesis of DN is the key to finding a new diagnosis and treatment scheme, but no significant breakthrough has been made yet (11). Evidence has shown that the pathological process of DN is related to renal fibrosis stimulated by various inflammatory factors (12). The vascular endothelial growth factor (VEGF), as a classic angiogenesis-inducing factor in the human body, is essential in promoting vascular permeability and extracellular matrix degeneration (13). In addition, it has been universally acknowledged in clinics that excessive release of VEGF can activate the release of a large number of inflammatory factors in the human body and cause a series of pathological changes of blood vessels, tissues and organs (14).

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In T2DM, VEGF shows aberrant expression (15), and is revealed to be closely related to the development of DN (16).

In the face of the increasingly high incidence of DN, finding a new diagnosis and treatment scheme has become a hot research project in clinics; whereas, the relationship between VEGF and DN remains to be further defined. Accordingly, this study probed into the expression of VEGF based on DN biopsy samples to confirm the role and clinical implications of VEGF in DN, so as to provide more in-depth and accurate guidance for follow-up clinical research.

Materials and Methods

Participants

Seventy-nine patients with suspected DN who underwent renal needle biopsy (RNB) in our hospital from January 2015 to June 2019 were selected as the research participants. The internal Ethics Committee ratified this study, and all participants provided informed consent before enrollment (Affiliated Hospital of Jiangnan University Ethic Committee, No. 2021001).

Eligibility criteria

DN patients: Confirmed diagnosis of DN by RNB; Complete case data; Age >18 years old; No drug allergic reaction or related contraindications. Exclude patients who have received antibiotics within 3 months before admission; Diabetic patients: T2DM with primary glomerulonephritis confirmed by RNB; Complete case data; Age >18 years old; No previous major medical history; Normal vital signs and organ functions.

PCR detection

Total RNA was extracted by the Trizol method, and cDNA was obtained by reverse transcription for PCR. The reaction conditions were 95℃ for 5s, 95℃ for 5s and 56℃ for 40s, for 40 cycles. The VEGF mRNA level was calculated by 2-ΔΔCt with GAPDH as an internal reference. Primer sequences are presented in Table 1.

Western blot detection

Total protein was extracted by BCA after renal tissue lysis. After electrophoresis and membrane transfer, the protein was sealed with 5%BAS for 1 hour and immersed in I antibodies for overnight cultivation at 4℃, following by incubation in the II antibody for 30 min the next day. After development using ECL, Image J was used to analyze the gray value of protein bands.

Follow-up for prognosis

DN patients were followed up for three years through hospital reexamination, and the recurrence was recorded.

Statistical methods

In this experiment, all statistical calculations were carried out with Prism 9 software. The categorical variables (n/%) were tested by the Chi-square test, and the continuous variables ($\chi \pm s$) were tested by independent sample one-way ANOVA and Duncan test, with $P<0.05$ as the significance level. The correlation was analyzed by the Pearson correlation coefficient, the predictive value by the ROC curve, and the related influencing factors by Logistic regression.

Results

Baseline characteristics of included patients

After the biopsy, 36 of them were diagnosed as DN, and the other 43 were T2DM combined with primary glomerulonephritis. The clinical baseline characteristics (age, sex, course of disease, etc. Table 2) were similar in DN patients and diabetic patients ($P>0.05$). No loss of follow-up and no dropout.

Table 1. Primer sequences.

	Direction	Sequence
VEGF	F	GGCC TCCGAAACCATGAACT
	R	TCGTGATGATT CTGCCCTCCG
GAPDH	F	GTCAAGG CTGAGAACGGGAA
	R	AAATGAGCCCCAG CCTTCTC

Table 2. Comparison of clinical baseline data.

	DN patients	Diabetic	t or χ^2	P
Age	50.8±6.6	49.6±7.2	0.766	0.446
Body mass index	22.8±4.9	23.4±5.0	0.536	0.594
Course of disease (year)	9.5±1.9	9.5±1.9	0.244	0.808
Fasting blood glucose (mmol/L)	10.81±2.06	10.42±1.84	0.889	0.377
Glycosylated hemoglobin (%)	7.81±2.40	7.51±1.98	0.609	0.544
Sex			0.087	0.768
Male *vs* female	31/5	36/7		
Family medical history			0.166	0.684
Yes *vs* no	11/25	15/28		
Smoking			0.212	0.645
Yes *vs* no	26/10	29/14		
Drinking			0.072	0.789
Yes *vs* no	22/14	25/18		
Exercise habits			0.005	0.943
Yes *vs* No	4/32	5/38		

VEGF expression

DN patients showed notably higher VEGF mRNA levels and protein expression in kidney tissue than diabetic patients (P<0.05). Fig. 1

Correlation between VEGF and kidney injury in DN patients

Pearson correlation coefficient analysis identified that VEGF mRNA level and protein expression in DN patients had a positive connection with blood urea nitrogen (BUN), serum creatinine (Scr) and 24-hour urine protein (24hUTP) (P<0.05). Fig. 2

Correlation between VEGF and inflammatory factors in DN patients

According to Pearson correlation coefficient analysis, VEGF mRNA level and VEGF protein expression also had a positive connection with inflammatory factor C-reactive protein (CRP) (P<0.05). Fig. 3

Correlation between VEGF and clinicopathological symptoms of DN patients

DN patients were divided into high and low VEGF groups based on median VEGF mRNA level. By comparison, we can see that there was no evident difference in age, BMI, sex, family history, smoking, drinking, exercise habits, clinical presentations manifestations and pathological changes between high and low VEGF groups (P>0.05), but the course of disease was longer and the fasting blood glucose (FBG), glycosylated hemoglobin (HBALC) and pathological stage of kidney disease were higher in high VEGF group (P<0.05) (Table 3).

Correlation between VEGF and prognosis of DN patients

During the 3-year follow-up, 32 out of the 36 DN patients were successfully followed up. DN recurred in 7 patients, with a total recurrence rate of 21.9%. As shown in Fig. 4, VEGF mRNA was markedly higher in relapsed patients than in non-relapsed patients (P<0.05). ROC curve analysis showed that when VEGF mRNA >5.20 in the kidney tissue of patients, the sensitivity and specificity for predicting the 3-year disease recurrence were 85.71% and 84.00% respectively (P<0.05).

Univariate analysis of prognostic recurrence of DN

As shown in Table 4, age, BMI, course of the disease, FBG, HBALC, renal pathological stage and VEGF level were the variables affecting the prognostic recurrence of DN patients (P<0.05).

Multivariate analysis of prognostic recurrence of DN

Indicators with differences in univariate analysis were assigned, and then substituted into SPSS for Logistic regression analysis (Table 5). As shown in Table 6, age, BMI and duration of disease, were not independent factors affecting the recurrence of DN (P>0.05), while FBG, HBALC and VEGF levels were (P<0.05).

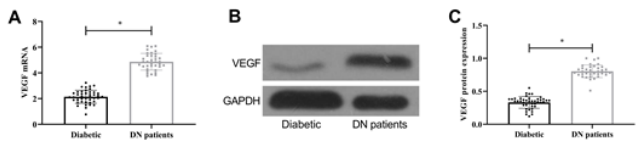

Figure 1. Expression of VEGF. (A) VEGF mRNA level; (B) Western blot; (B) VEGF protein expression. Note: * P<0.05.

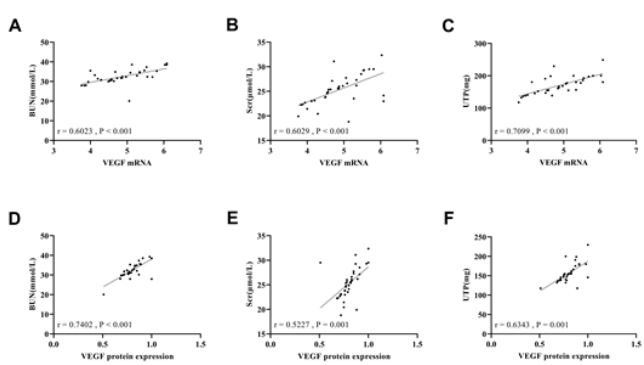

Figure 2. Correlation between VEGF and kidney injury in patients with DN. (A) Correlation between VEGF mRNA and BUN; (B) Correlation between VEGF mRNA and Scr; (C) Correlation between VEGF mRNA and 24hUTP; (D) Correlation between VEGF protein expression and BUN; (E) Correlation between VEGF protein expression and Scr; (F) Correlation between VEGF protein expression and 24hUTP.

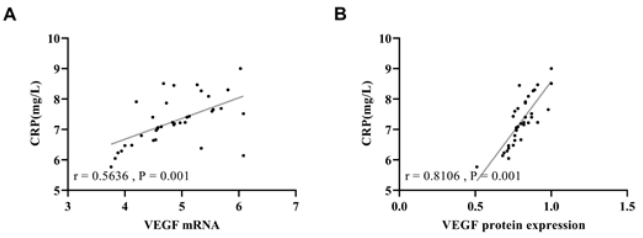

Figure 3. Correlation between VEGF and inflammatory factors in patients with DN. (A) Correlation between VEGF mRNA and CRP; (B) Correlation between VEGF protein expression and CRP.

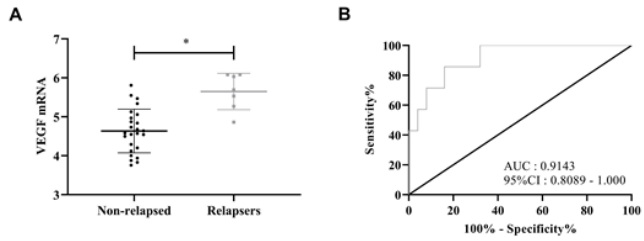

Figure 4. Relationship between VEGF and prognosis of DN patients. (A) VEGF mRNA in patients with recurrence and those without. Note: * P<0.05. (B) ROC curve of predicting prognosis and recurrence of DN by VEGF mRNA level in kidney tissue.

Discussion

As one of the most common chronic diseases of T2DM, DN has a great potential threat to patients (17). Kidney transplantation is the most ideal and effective treatment for DN (18). However, the popularity of organ donation in clinics is not high at present, so it is often difficult for DN patients to obtain a matching allogeneic kidney for transplantation (19). Moreover, T2DM can not be cured completely, and DN patients may experience disease relapse caused by T2DM (20). Studies have shown that the underlying pathophysiological process leading to DN is the

Table 4. Univariate analysis of prognostic recurrence of DN

	Relapsers	Non-relapsed	t or χ^2	P
Age	54.2±4.8	46.7±5.1	3.479	0.002
Body mass index	24.12±1.84	20.14±1.09	7.295	<0.001
Duration of disease (year)	13.84±0.89	8.40±1.04	12.570	<0.001
Fasting blood glucose (mmol/L)	12.84±0.87	8.89±0.64	33.620	<0.001
Glycosylated hemoglobin (%)	10.14±0.86	6.21±1.89	5.301	<0.001
Sex	5.65±0.47	4.63±0.56	4.391	<0.001
Male *vs* female			0.026	0.872
Family medical history	6/1	22/3		
Yes *vs* no			0.030	0.863
Smoking	2/5	8/17		
Yes *vs* no			0.030	0.863
Drinking	2/5	8/17		
Yes *vs* no			0.110	0.741
Exercise habits	4/3	16/9		
Yes *vs* No			0.026	0.872
Clinical presentations	1/6	3/22		
Acute renal failure *vs* hematuria *vs* proteinuria			0.305	0.859
Pathological changes	4/2/1	17/5/3		
Membranous nephropathy *vs* IgA nephropathy *vs* interstitial nephropathy			0.033	0.983
Renal pathological stage	5/1/1	18/4/3		
Type IIa *vs* type IIb *vs* type III *vs* type IV			9.895	0.020
	0/0/2/5	6/8/7/4		

Table 5. Single factor assignment.

Factor	Assignment
Age, BMI, Course of disease, Fasting blood glucose, Glycosylated hemoglobin, VEGF mRNA	The data conforms to continuous variables, and the original data is used for analysis
Renal pathological stage	Type IIa=1; Type IIb=2; Type III=3; Type IV=4

Table 6. Multivariate analysis of prognostic recurrence of DN.

Factor	B	S.E.	OR	Wald	P	95%CI
Age	2.304	0.668	1.842	2.542	0.241	1.042-7.862
BMI	1.008	0.543	0.211	3.524	0.194	0.115-0.465
Course of disease	2.124	1.242	1.567	3.954	0.150	0.621-1.824
Fasting blood glucose	1.254	0.318	1.548	16.524	0.000	1.084-5.162
Glycosylated hemoglobin	1.245	0.476	2.512	6.854	0.012	2.184-4.628
Renal pathological stage	0.724	0.941	1.242	2.424	0.251	0.642-1.841
VEGF mRNA	1.862	0.841	4.242	8.424	0.004	1.180~14.852

change of metabolism and hemodynamics (21), which has laid a foundation for the study of VEGF in DN in recent years. Although VEGF has been proven to be abnormally expressed in DN (22), its relationship with pathological changes remains uncharacterized. This study, through the exploration of VEGF, carries huge clinical implications for follow-up research.

First, we found that VEGF increased remarkably in the kidney tissue of DN patients, which was consistent with the results of previous literature (23). VEGF, as a classic proangiogenic growth factor, was first proven to have a strong regulatory effect on neovascularization (24). With the deepening of research, it has also been found to promote cell chemotaxis and mitosis, vascular penetration, and induce proliferation of lymphatic endothelial cells (25). In many neoplastic diseases, VEGF increases the pressure of tumor stroma by increasing lymphatic permeability to promote tumor infection (26, 27). As we all know, the development of DN is a process of aggravating the inflammation and fibrosis of kidney tissue (28). After the onset of T2DM, the body's internal environment is in a state of high glucose for a long time, which will form obvious microcirculation disturbance, leading to pseudohypoxia and ischemia in tissues; while the kidney is one of the organs that are extremely sensitive to ischemia and hypoxia, which directly causes severe stress reaction of the kidney (29, 30). At this time, renal vascular endothelial cells secrete a large amount of VEGF to promote the

compensatory hyperplasia of new blood vessels, fibrous ducts and lymphatic vessels to relieve ischemia and hypoxia, while enhancing the permeability of blood vessels. As such, a large number of inflammatory factors can penetrate into various organs and tissues of the body along with blood circulation, causing more serious diseases (31). The above is also a preliminary analysis of the mechanism of VEGF in DN combined with previous studies.

Then, through correlation analysis, we found that VEGF level in patients with DN was positively correlated with BUN, Scr, 24hUTP and CRP, which indicated that VEGF showed an increasing trend with the progression of DN. The reason may be the same as our above inference. The kidney of DN patients is seriously damaged, and the amount of VEGF secreted by renal vascular endothelial cells is increased (32), forming a vicious circle that continuously accelerates the development of DN. In subsequent studies, we found that the course of the disease was longer and the FBG, HBALC and pathological stage of kidney disease were higher in high VEGF group *vs* low VEGF group, which also verified our above viewpoint, indicating that the more severe the DN, the higher the VEGF level. Finally, in the follow-up of prognosis, we found that VEGF had a certain predictive value for the recurrence of DN, which also suggested that we can monitor the VEGF of patients in future clinical practice to timely understand the recovery of patients. At this stage, no effective specific blood marker of DN has been found clinically, nor were there any special symptoms in the early stage of DN, resulting in the difficulty in timely identifying the disease (33). The application of VEGF may greatly improve the clinical diagnosis rate of early DN, and timely intervene to reduce the risk of end-stage renal disease. Through Logistic regression analysis, we found that FBG, HBALC and VEGF levels were independent risk factors affecting the recurrence of DN, which further illustrated the importance of VEGF in DN. For various refractory diseases in the clinic, the attention is gradually shifted to targeted therapy from the molecular perspective (34, 35). Our experimental results also preliminarily suggest that we may achieve better results than the current clinical treatment of DN by targeted inhibition of VEGF expression in the future, thus bolstering the prognosis of patients.

Although this experiment has made a preliminary analysis of the role of VEGF in DN, there is still room for improvement. First of all, this study is based on kidney tissue samples of DN patients after RNB, while BUN, Scr and CRP are routine items of admission examination in our hospital; Since we have not collected blood samples of patients for correlation analysis, we cannot infer the preliminary diagnostic value of VEGF for DN. In addition, due to the lack of support from *in vitro* experiments, the mechanism of VEGF's participation in DN proposed above has not yet been verified. Based on the above limitations, we will arrange and conduct supplementary research as soon as possible, hoping to contribute to the new molecular diagnosis and treatment strategy of DN.

VEGF level in the kidney tissue of DN is closely linked to the renal function of patients and increases with the progression of the disease, which is an independent risk factor affecting the prognosis and recurrence of DN, with huge potential for DN diagnosis and treatment.

Declarations

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contributions

Jing Wu designed the study and wrote the manuscript, Junlin Zhang wrote and revised the manuscript, Huixian Zhu collected and analyzed data, and Qiong Tang supervised the research. All authors read and approved the final submitted manuscript.

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Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Affiliated Hospital of Jiangnan University (Approve number: LS2021001).

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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